Open Access
J. Glob. Innov. Agric. Sci., 2025, 13(1):85-94
ISSN (Online): 2788-4546; ISSN (Print):2788-4538
DOI: https://doi.org/10.22194/JGIAS/25.1528
http://www.jgiass.com

Estimation of Indole-3-Acetic Acid Production from New Molecularly Characterized Rhizobacterial Strains

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From different fields in Mosul city, we collected sixteen samples of soil surrounding the rhizosphere zone to isolate and characterize rhizobacterial species based on biochemical tests, *16S rRNA* gene primer amplification using PCR and nucleotide sequence analysis, and similarity to global isolates in the gene bank. All isolates were bacilliform, with clear differences in their response to Gram staining and biochemical tests. On the other hand, the results showed that root colonizing bacteria could produce different amounts of indole-3-acetic acid (IAA). Molecular analysis tests based on the *16S rRNA* primer gene were carried out to characterize the isolated bacteria at the molecular level and showed 99% homology with *Azotobacter tropicalis* SC39, *Azotobacter chroococcum* A11, *Bacillus subtilis* N22 and *Ralstonia pickettii* ULM005, which are registered worldwide in GenBank. It should be noted that in the diagnostic isolate *R. pickettii* both A and T were deleted, G was replaced by C and T was added at position 508. On the other hand, in the *B. subtilis* isolate, the nitrogenous bases A, G and G were deleted and the nitrogenous base G was replaced by the base C. The data for the third isolate, *A. tropicalis*, showed deletion of the nitrogenous bases C and T and replacement of G by A and A by T. In the fourth isolate, *A. chroococcum*, deletion of the nitrogenous base, replacement of C by A and C by T and addition of G, T and A in three positions were observed. These will be used as the basis for future scientific experiments to develop new biofertilizers from the rhizobacteria studied for the production of environmentally sustainable crops.

Keywords: Biochemical testes, IAA, molecular identification, nucleotide sequence, Rhizobacteria.

INTRODUCTION

Rhizosphere bacteria that positively influence plant growth and productivity of commercially important crops are commonly referred to as Plant Growth Promoting Rhizobacteria (PGPR) and include bacteria of the genera *Azotobacter, Azospirillum, Arthrobacter, Bacillus, Agrobacterium, Rhizobium, Flavobacterium, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Xanthomonas* and *Serratia*. The secretion of root exudates helps regulate microbial dynamics and their interactions with plants, and in turn these bacteria play an important role in promoting plant growth. In addition, such symbiotic associations in the rhizosphere also confer protection against various diseases caused by fungal, bacterial and viral pathogens .These bacteria directly affect plant growth through the production and secretion of growth-promoting substances such as auxins, gibberellins and cytokinins, by stimulating root metabolic activities using bacterial surface components and/or by providing biologically fixed nitrogen (Dutta *et al.*, 2022;Karthikeyan *et al*, 2021). Approximately 80% of microorganisms in the root zone are capable of synthesizing and releasing auxin as a secondary metabolite (Kurrey *et al.*, 2018; Ratnaningsih *et al.*, 2023), which regulates many processes in plant tissues, particularly those important for plant growth and development (Zhu *et al.*, 2020), particularly its direct effect on lateral root formation and promotion of root hair growth (Batista *et al.*, 2021).

Ralstonia pickettii QL-A6, a low virulence gram-negative bacillus, was isolated from the rhizosphere of tomato plants and successfully used to suppress bacterial wilt of tomatoes caused by *Ralstonia solanacearum* (Wei *et al.*, 2013; Wang *et al.*, 2024).

Many researchers have indicated that the most common bacterial species around roots isolated from the rhizosphere are bacteria belonging to the genus *Bacillus*, and the most famous Gram-positive species are *Bacillus cereus* and *Bacillus subtilis* (Arora, 2020), and are considered one of the

Harbawi, M.A., N.I.K. Al-Barhawee and F.A. Al-Wazzan. 2025. Estimation of Indole-3-Acetic Acid Production from New Molecularly Characterized Rhizobacterial Strains. Journal of Global Innovations in Agricultural Sciences 13:85-94.
[Received 2 Sept 2024; Accepted 6 Oct 2024; Published 1 Jan 2025]

most important plant growth promoting bacterial species (PGPR) due to their effectiveness in dissolving phosphates used as biofertilizers (de Andrade *et al*., 2023), in addition to their ability to produce iron-soluble siderophores and auxin phytohormone indole-3-acetic acid (IAA). They grow well on nutrient agar and can be motile with or without motile flagella (Losick, 2020).

In a study Hanoush (2023), *Bacillus* spp. were isolated from local juices in the city of Ramadi, and the most common species was *Bacillus cereus*, followed by *Bacillus subtilis*, and four species, *B. megaterium*, *B. subtilis*, *B. circulans* and *B. licheniformis*, were isolated from sterilized milk produced by the Mosul Dairy (Muhammed, 2005).

Free-living (non-symbiotic) bacteria with the ability to fix molecular nitrogen belong to the genera *Azotobacter* and *Azospirillum* and are members of the family Azotobacteraceae. They produce growth-promoting substances that improve seed germination and root system growth on a large scale, and produce various sugars that improve soil aggregation (Patel and Bhatt, 2011). *Azotobacter* is a group of Gram-negative, free-living, nitrogen-fixing, aerobic, soil-colonizing rhizobacteria, often with a short bacillus or acroporoid shape, characterized by the formation of thick-walled cysts under unfavorable environmental conditions for their growth, on the other hand, stress factors (biotic and abiotic) generally lead to impaired plant germination and reduced productivity. Among the plant growth promoting rhizobacteria, *Azotobacter* spp. provide plants with phytohormones such as indole-3-acetic acid production, avoid various stresses, fix atmospheric nitrogen, degrade pesticides and oil globules, metabolise heavy metals, etc.

The current review aims to assess the usefulness of *Azotobacter* spp. in improving plant health in a sustainable agroecosystem (Sumbul *et al*., 2020).

Azotobacter tropicalis inhibits the growth of saprophytic and pathogenic microorganisms near the root system of field crops. In vitro, it has been shown to produce indole acetic acid when tryptophan is added as a substrate to the culture medium (Patel and Bhatt, 2011), while research on *A. chroococcum* has shown its importance in improving plant nutrition and soil fertility (Wani *et al*, 2013), and the substances it produces can be involved in various processes leading to improved plant growth (Jnawali *et al.,* 2015), and its use in research experiments as a microbial inoculant has significantly improved crop production in agriculture (Gothandapani *et al.,* 2017).

Advances in DNA sequence analysis techniques, sequencing of conserved bacterial genomic regions, and comparison with Genbank sequences have led to the identification of bacteria by genus with high accuracy and have become the preferred method for rapid bacterial identification and diagnostic confirmation (Franco-Duarte *et al.,* 2019). Therefore, molecular analysis has shown that rhizobacteria share up to 99.4% homology with *Bacillus subtilis* DQ198162.1 (Wicke *et al.,* 2023) and are diagnosed as *Azotobacter chroococcum* when compared to the global standard strain in the *Azotobacter chroococcum* CAZ3 Genebank (Rizvi *et al.,* 2019), and are diagnosed as *R. pickettii* based on their identity to the globally recorded *R. pickettii* NCTR106 isolate (Kim *et al.,* 2022).

Molecular characterization of bacteria provides more accurate information than traditional techniques, especially as the amplification of generic primers has become a reference for further molecular research. Therefore, we studied and evaluated the evolutionary variants of the 16S rRNA gene in three rhizosphere-endemic bacterial species, based on the comparison of the sequence of this gene with that of several species registered in GenBank, and evaluated their efficiency in producing the main plant growth regulator, IAA, so that the results of this study can be used as plants growth-promoting factors within the improvement of sustainable agriculture in the future.

MATERIALS AND METHODS

***Soil samples*:** Soil rhizosphere samples of different plants were collected from the fields of; Agriculture and Forestry College / Mosul University, Agriculture College / Tikrit University, Agricultural Protection Directorate, and Al-Rashidiya area.

Plant samples were collected together with the soil attached to them and transported to the laboratory in sterile containers. In the sterile cabinet, we shook the roots of the plants to remove loose soil, while a sterile brush was used to collect the remaining soil (Bulgarelli *et al*., 2013).

***Bacterial isolation*:** A series of six dilutions (10^{-1} - 10^{-6}) of the soil samples were made in sterile 10 ml test tubes containing 9 ml of sterile distilled water (Mahato *et al.,* 2021). After numbering, some tubes were placed in a water bath at a temperature of 80 degrees Celsius for one hour to isolate the spore-forming bacteria and the rest of the tubes were not exposed to heat to isolate the non-sporeforming bacteria. 1 ml of the last three dilutions were transferred and cultured on nutrient agar and incubated at 28 degrees Celsius for two days. Individual colonies were selected and stored for testing on a solid slant medium at 4 degrees Celsius, while isolates were stored for longer periods at -60 to -80 degrees Celsius on a liquid nutrient medium supplemented with sterile glycerol.

***Diagnosis of isolated bacteria*:** Diagnosis was based on the microscopic cultural characteristics of the bacterial isolates, as well as biochemical and molecular tests.

***Cultural characteristics*:** The isolated bacteria were grown on a nutrient agar culture medium using the streaking method and incubated at a temperature of 28±2 degrees Celsius for 24 hours. The cultural characteristics of the colonies growing on the solid medium were then examined, including colony

surface, shape, texture, color, brightness, pigment production, and edge shape according to Mahon and Manuselis (2000).

***Microscopic characteristics by Gram staining*:** Smears of bacterial isolates were prepared on glass slides to determine the response of bacterial isolates to this stain. They were examined under a light microscope according to Collee *et al.* (1996) at 100X magnification to determine the shape of the cells, the way they were grouped , their ability to take the stain, and spore formation**.**

***Biochemical tests*:** A series of biochemical tests were performed on the studied isolates, consisting of the following: bacterial motility (Nishihara and Freese, 1975), Congo red dye test (Cimdins and Simm, 2017), growth at 37°C (Petrillo *et al*., 2020), catalase, oxidase, and IMViC tests (Holt *et al.*, 1996), urease (Collee *et al.*, 1996), starch hydrolysis test (De Oliveira, 2016), and triple sugar iron agar (TSI test).

***Determination of the ability of the isolates to produce indole-3-acetic acid and estimation of its concentration*:** The efficiency of the rhizobacterial isolates in the production of indole-3-acetic acid (IAA) was determined by the colorimetric method (Patten and Glick, 1996) using autoclaved sterile peptone-water medium, followed by incubation of the medium at 28 degrees Celsius for 24 hours after inoculation with the bacterial isolates. The inoculated medium was centrifuged at 3000 rpm for 15 minutes and 1 ml of pre-prepared Salkovsky's reagent was added to the cell-free supernatants, then the mixture was left in complete darkness at 25-30 degrees Celsius for 30 minutes and the absorbance was measured at a wave length of 535 nm using a spectrophotometer. The concentration of IAA in each isolate was calculated by projecting the absorbance values onto the IAA standard curve (Fig. 1).

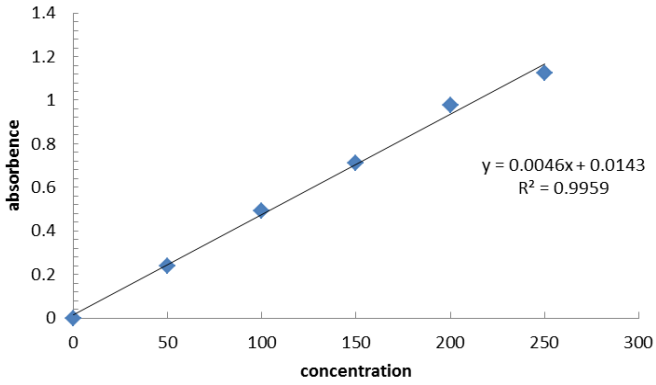

Figure 1. Indole acetic-3- acid (IAA) standard curve.

***Molecular diagnosis*:** Four rhizobacterial isolates studied (Z4, Z7, Z11 and Z16), which were the most productive producers of IAA, were selected and characterized by molecular PCR and the use of *16S rRNA* gene primers, then comparing the results of the sequence of nitrogenous bases of the DNA isolated from these bacteria with the sequence recorded in the database belonging to GenBank to identify the bacterial isolate at the genus, species, and strain levels (Altaai *et al.*, 2014).

***Extraction of genomic DNA*:** By using a DNA extraction kit (DNA Purification Kit/GENEAID) containing the reaction components of both the polymerase chain reaction (PCR) and the master mix, genomic DNA was extracted according to the steps below and as indicated by the Korean company that manufactures it:

Bacteria were grown in liquid media and centrifuged. Then 1 x 10^9 cells of the sediment were transferred to a 1.5 ml Eppendorf tube, and 200 µl of the enzyme lysozyme at a concentration of 0.8 mg/20 ml was added. The mixture was then mixed well using a vortex device and the tube was incubated at 37 degrees Celsius for 30 minutes, taking care to invert the tube every 3 minutes, then 20 µl of protease K enzyme was added and incubated at 60 degrees Celsius for 10 minutes. Then 200 µl of GB buffer solution was added, mixed well by vortexing, and incubated at 70 degrees Celsius. Then 200 µl of ethanol was added and centrifuged at 16,000 rpm for 30 seconds, the floating portion was discarded and 600 µl of wash solution was added to the precipitate. To remove traces of the wash solution, the mixture was centrifuged at the same time and speed as above. The sedimentary fraction was collected in the GD column, then its contents were transferred to a 1.5 ml Eppendorf tube, 100 µl of the dissolving solution was added and the mixture was centrifuged for 30 seconds at 16,000 rpm to complete the precipitation of the DNA, which was stored at -20 degrees Celsius for later use.

***Primers*:** The most commonly used genetic marker for identification and strain identification is the 16S ribosomal RNA gene sequence, which is used to study bacterial phylogeny and taxonomy. PCR was performed on the *16S rRNA* gene using universal primers designed by Lane (1991), whose sequences are shown in Table 1, to identify four selected rhizobacteria isolated from the rhizosphere of different plants.

Table 1. 16S rRNA primer sequence.

16S rRNA primers	Sequence	Product size(pb)
27F	5'- AGAGTTTGATCCTGGCTCAG-3'	1250
1492R	5'- GGTTACCTTGTTACGACTT-3’	

***Preparation of the PCR reaction*:** The Applied Biosystem Gene-amp PCR System 9700 thermal polymerase device was used in the gene replication process to amplify the gene *16S rRNA* by using 10 ng of genomic DNA isolated from bacterial strain, and the PCR product was separated by electrophoresis on a 1.5% agarose gel according to the standard reaction conditions shown in Table 2 after treatment with Intron Korea Red stain and UV irradiation, bands of genetic DNA appeared at a wavelength of 302 nm and were photographed using a digital camera.

Table 2. Standard conditions for the PCR reaction.

No.	Phase	Tm(˚C)	Time	No. of cycle
1	Initial Denaturation	95	5 min	1
2	Denaturation	95	45 sec	35
3	Annealing	58	45 sec	
4	Extension -1	72	45 sec	
5	Extension -2	72	7 min	1

***Determine the nucleotide sequence*:** After obtaining the DNA amplification product, the nucleotide sequences of the amplified *16S rRNA* gene were determined by sending a volume of 25 microlitres of the PCR product and a volume of 10 microlitres (concentration of 10 picomoles) of primer to the Korean company Biotechnology Lab, using the Applied Biosystem 3730XL DNA sequencer, and using a computer program linked to the Basic Local Alignment Search Tool (BLAST), the results were compared with the database at the National Center for Biotechnology Information (NCBI), where the percentage of similarity with international varieties registered in the GeneBank was determined according to the international number assigned to them.

RESULTS AND DISCUSSION

Conventional tests for diagnosis of isolated bacteria: The results of conventional tests showed that the bacteria isolated in this study from different regions of the rhizosphere were bacilliform, motile, but varied in their acceptance of Cram's dye, capture of Congo red dye, spore formation (Fig. 2), salinity resistance, ability to grow at 37 degrees Celsius, production of catalase, oxidase, urease and amylase enzymes, TSI test results and IMViC assays (Table 3). Some of these results were consistent with those of Errington and Aart (2020) in their diagnostic results for *Bacillus subtilis* as a fast-growing, aerobic, Gram-positive bacterium with rod-shaped cells, typically 2-6 µm in length and less than 1 µm in diameter, and the growth temperature is around 30-35 degrees Celsius, with a generation time of up to 20 minutes under optimum conditions, while under starvation conditions, the cells can undergo a differentiation process leading to the formation of endospores. Vegetative cells can be motile and form biofilms. One edition of Bergey's Manual identified 141 species of *Bacillus* (Zeigler and Perkins, 2021), using a wide range of characteristics to distinguish *B. subtilis* from other species within the genus *Bacillus*, in particular, the types of murein cross-bridges (peptidoglycan); the ability to hydrolyze and utilize different carbon sources; the shape of their colonies; the ability of their cells to form spores; and their tolerance to changes in salinity, pH and temperature.

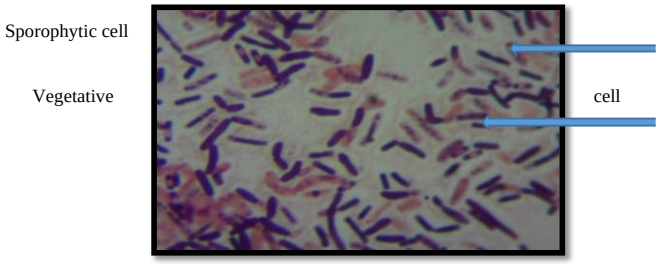

Figure 2. Saprophytic and vegetative cells of rhizobacterial isolated.

***IAA production by bacteria*:** The results also show the ability of bacteria isolated from the rhizosphere of plants from different areas to produce different amounts of IAA. The most productive rhizobacteria (Z_{11}) were isolated from the rhizosphere of the Garlic plant (2.51 µg/ml), followed by rhizobacteria (Z_{16} and Z_7) isolated from the rhizosphere of the Local Beans plant (2.38 µg/ml), the rhizosphere of the red clover plant (2.28 µg/ml) and finally rhizobacteria isolated from the rhizosphere (Z_4) of the Okra plant (2.12 µg/ml), as

Table 3. Results of traditional tests for bacteria isolated from the different fields.

Transactions	location	Isolation source	Gram stain	Cell shape	Motility	Congo red dye	Sporulation	Salinity	Growth at 37°C	Catalase test	Oxidase test	Urease test	Starch hydrolysis test	TSI test	IMViC tests			
															Indole	Methyl red	Voges-Proskauer	Citrate
z_1	College of Agriculture and Forestry University of Mosul	Potatoes	-	Rod	+	-	+	+	+	+	+	+	+	+	-	+	-	-
z_2		Bean	-	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	-
z_3		Okra	+	Rod	+	+	+	+	+	+	+	+	+	+	-	+	-	+
z_4		Apium	+	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+
z_5		Cucumber	+	Rod	+	+	+	-	+	-	+	_	+	-	-	-	-	+
z_6		Cucurbita	+	Rod	+	+	-	-	-	-	+	+	-	-	+	+	+	+
z_7	Fields of the College of Agriculture Tikrit University	Red clover	+	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+
z_8		Wild clover	-	Rod	+	+	+	+	+	+	+	+	+	+	-	+	-	-
z_9		Zea mays	+	Rod	+	-	+	+	+	+	+	+	+	+	+	+	+	-
z_{10}	Agricultural Protection Directorate	Onion	+	Rod	+	-	-	+	-	-	+	-	-	+	+	-	+	+
z_{11}		Garlic	+	Rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+
z_{12}		Lettuce	+	Rod	+	+	+	-	+	-	+	+	+	-	-	+	-	+
z_{13}		Holland beans	+	Rod	+	+	+	-	+	-	+	-	+	-	-	-	-	+
z_{14}	Errachidia area	Chard	-	Rod	+	+	+	+	+	-	+	+	+	+	-	+	-	+
z_{15}		Onion	-	Rod	+	-	+	+	-	+	+	+	+	+	+	+	+	+
z_{16}		Local beans	+	Rod	+	+	+	+	+	+	+	+	+	+	-	+	+	+

shown in Fig. 3. Statistical analysis (Table 4) showed significant differences in the production of IAA by the bacteria studied.

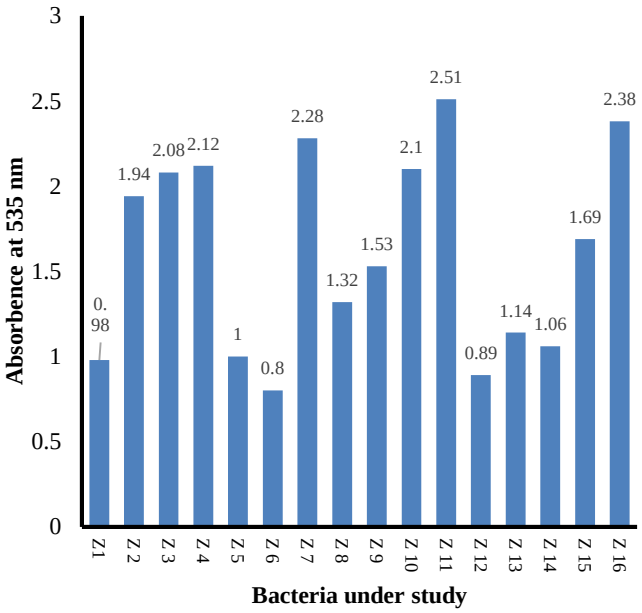

Figure 3. IAA (µg/ml) produced by rhizobacterial strains isolated from the rhizosphere of different plants.

Table 4. Results of the statistical analysis.

Code of bacteria	Absorbance (535 nm)	t - grouping
Z1	0.98	EF
Z2	1.94	ABC
Z3	2.08	ABC
Z4	2.12	AB
Z5	1.00	EF
Z6	0.80	F
Z7	2.28	A
Z8	1.32	DEF
Z9	1.53	CDE
Z10	2.10	ABC
Z11	2.51	A
Z12	0.89	F
Z13	1.14	DEF
Z14	1.06	EF
Z15	1.69	BCD
Z16	2.38	A

Data are the average of three replicates, similar letters; no significant differences between them and the different letters; there are significant differences between them.

A total of 21 free-living diazotrophs were isolated from different rhizospheric soils of North Gujarat. Among them, a single isolate was selected for the production of indole acetic acid (IAA) in a medium containing 1, 2 and 5 mg/ml of tryptophan. The isolate showed high production (31.4 mg/l) of IAA at 5 mg/ml of tryptophan while at 1 and 2 mg/ml the production was 8.76 and 18.92 mg/l respectively (Patel and Bhatt, 2011). *Rhizobium undicola* strain N37, isolated from the aquatic legume *Neptunia oleracea*, was found to produce abundant IAA in YEM broth medium supplemented with tryptophan, reaching a plateau after 20 hours, enrichment of the medium with mannitol, ammonium sulphate, B12 and 4-hydroxybenzaldehyde was found to enhance IAA production (Ghosh *et al.*, 2015). IAA production can be optimized by altering the growth conditions of bacterial isolates to improve plant growth as an environmentally friendly method.

Rhizobacteria identified as *Bacillus siamensis* produced high levels of IAA (10.23 and 16.61 µg/ml) in the presence of sucrose and try tone as carbon and nitrogen sources, whereas the maximum production of IAA (8.42 and 13.29 µg/ml) was at 35 degrees Celsius and pH 8.0, respectively (Widawati, 2020). The low levels of IAA in this study may be due to the high levels of IAA catabolic enzymes such as IAA oxidase and IAA peroxidase (Ghosh *et al.*, 2015).

***Molecular diagnosis of bacterial isolates based on 16SrRNA gene primers*:** The exposed genomic DNA bands were visualized by UV light (Figure 4), indicating that these bands were characterized by their large size as they travelled short distances on the agarose gel. When the purified genomic DNA from the four bacterial isolates studied was analyzed using the universal *16S rRNA* primer and DNA ladder, it was found to be 1250 bp in size (Fig. 4).

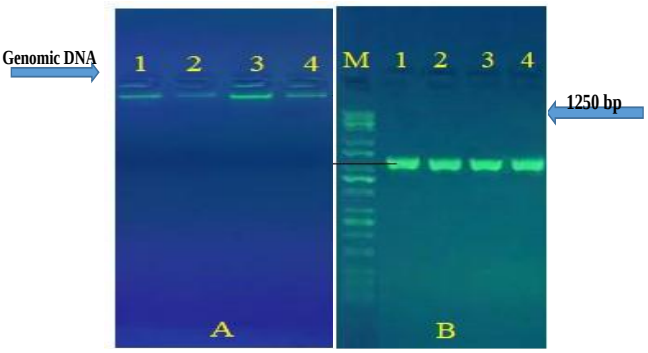

Figure 4. Electrophoresis of the polymerase chain reaction product of four rhizobacteria genomic DNA (A), using universal primers for the *16S rRNA* gene (B).

M: DNA Ladder Volume = 1Kb; 1, 2, 3 and 4: genomic DNA of Z4, Z7, Z11 and Z16 rhizobacteria, respectively.

Genome sequence data from the National Bureau of Bacterial Information database (//ncbi.nlm.nihv.gov. Genomes), shown in Figures (5-8) and Table 5, confirmed that all rhizobacterial species isolated from the rhizosphere in this research are 99% identical with the standard isolates *Ralstonia pickettii* ULM005, *Bacillus subtilis* N22, *Azotobacter tropicalis* SC39, and *Azotobacter chroococcum* A11, for isolates Z_4, Z_7, Z_{11}

and Z_{16}, consecutively. It should be noted that the nitrogenous bases in the diagnostic isolate *Ralstonia pickettii* had both A and T deleted at positions 156 and 466 respectively, G replaced by C at position 234 and T added at position 508. On the other hand, in the isolate registered in GenBank as *Bacillus subtilis* strain MN-Mosul Univ. 3 16S ribosomal RNA, partial sequence, the nitrogenous bases A, G and G were deleted at positions 147, 155, 324 and the nitrogenous base G was replaced by base C at position 281.The data for the third isolate, *Azotobacter tropicalis*, showed deletion of the nitrogenous base C at position 356 and T at position 417, and substitution of G for A and A for T at positions 455 and 812, respectively. In the fourth isolate, *Azotobacter chroococcum*, deletion of the nitrogenous base G at position 155, substitution of C for A and C for T at positions 311 and 291, and addition of G, T and A at positions 290, 400 and 581 were observed.

A rapid method with high sensitivity and specificity has been developed for the molecular identification of isolated bacteria, such as the analysis of the *16S rRNA* (16S ribosomal RNA) gene. It is widely used because it is multicopy, universal in bacteria and conservative It can be used as a discriminator between species, and the bacterial database based on primer amplification of the *16S rRNA* gene is extensive, making comparisons between different isolates of bacteria very easy (Akihary and Kolondam, 2020).

A study Ryan *et al.* (2011) using species-specific PCR showed that fifteen of fifty-nine *R. pickettii* isolates diagnosed by conventional methods were in fact closely related to *R. insidiosa*, and PCR profiling of *16S-23S rRNA* showed few significant differences between isolates. High *16S rRNA* gene sequence similarity (100%) was found between the isolate and *R. pickettii* strain ATCC 27511 (GenBank accession number JOVL01000020), while contiguous dendrograms based on *16S rRNA* gene sequences showed that this isolate belongs to the genus *Ralstonia* and forms a subgenus with *R. pickettii* ATCC 27511 (Li *et al*., 2019). When the *16S rRNA* gene was amplified in a set of *B. subtilis*, its size was 1550 bp (Matar *et al*, 2009), while its size in wild-type rhizobacteria isolated from soil was 1522 bp compared to the wild-type *B. subtilis* BB1 standard (Al-Rubyee and Al-Barhawee, 2023). These results are in line with the findings of Devereux and Wilkinson (2004), who used molecular techniques and primer amplification of the *16S rRNA* gene with template DNA isolated from several strains belonging to *B. subtilis* bacteria to diagnose B. subtilis isolated from soil by culture, microscopic methods, as well as biochemical assays. When comparing the results with the standard strains contained in the National Center for Biotechnology Information (NCBI), they found that the similarity rate was 98 and 99%. In contrast, in a different study, they had percentages of 100, 100, 99.9, 99.9, 99.9, 99.8 and 99.8, were identical to *B. subtilus* strains KCCM 32835, PS832, HRBS-10TDI13, GFR-12, 2RL2-3, and SRCM102748, respectively (Lee *et al.*, 2022). The DNA/BLAST program was used by researchers Al-Rubyee and Al-Barhawee (2023) to confirm that the wild standard isolate *Bacillus subtilis* strain BB1 and the three local isolates, Sar1, Sar2, and Sar3, had similarity percentages of 98.19, 98.12, and 94.94%, respectively.

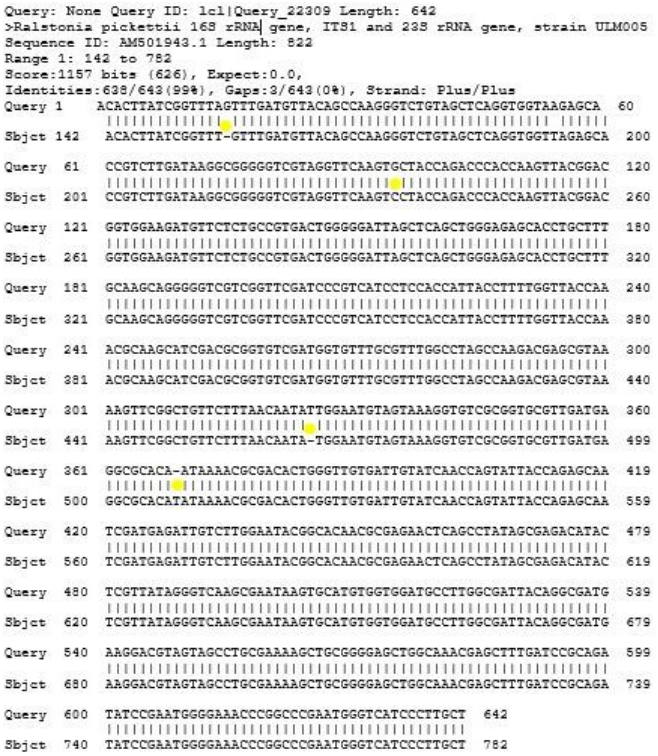

Figure 5. Nucleotide sequence alignment of the *16S rRNA* gene of the Z4 bacteria with the closest match to the *Ralstonia pickettii* ULM005 registered in GenBank.

Table 9. Molecular diagnosis of bacterial isolates based on the percentage of identity of *16S rRNA* gene sequences with bacterial strains registered in the World Gene Bank on the NCBI website.

Isolation No.	The most identical bacteria	World number	Country	Similarity ratio (%)	Registering bacteria in the geneBank
Z_4	*Ralstonia pickettii strain ULM005*	AM501943.1	France	99.22	OP740520.1
Z_7	*Bacillus subtilis* strain N22	OP536008.1	China	99.43	OP740401.1
Z_{11}	*Azotobacter tropicalis* strain SC39	ON261598.1	Turkey	99.52	OP740399.1
Z_{16}	*Azotobacter chroococcum* strain A11	OL636178.1	Serbia	99.34	OP740408.1

The researchers also found that the difference between the first and second isolates only occurred at position 464 of the base, where the nitrogenous base G of the isolate Sar1 and the nitrogenous base A of the isolate Sar2, resulting in a 99.93% similarity between them. On the other hand, a total of 98 *Azotobacter* isolates were obtained from 27 rice rhizosphere soils of Taiwan , and *16S rRNA* gene sequences were used to identify *Azotobacter* species such as *A.beijerinckii* CHB 461, *A. chroococcum* CHB 846, and *A. chroococcum* CHB 869 (Chen *et al.*, 2018).

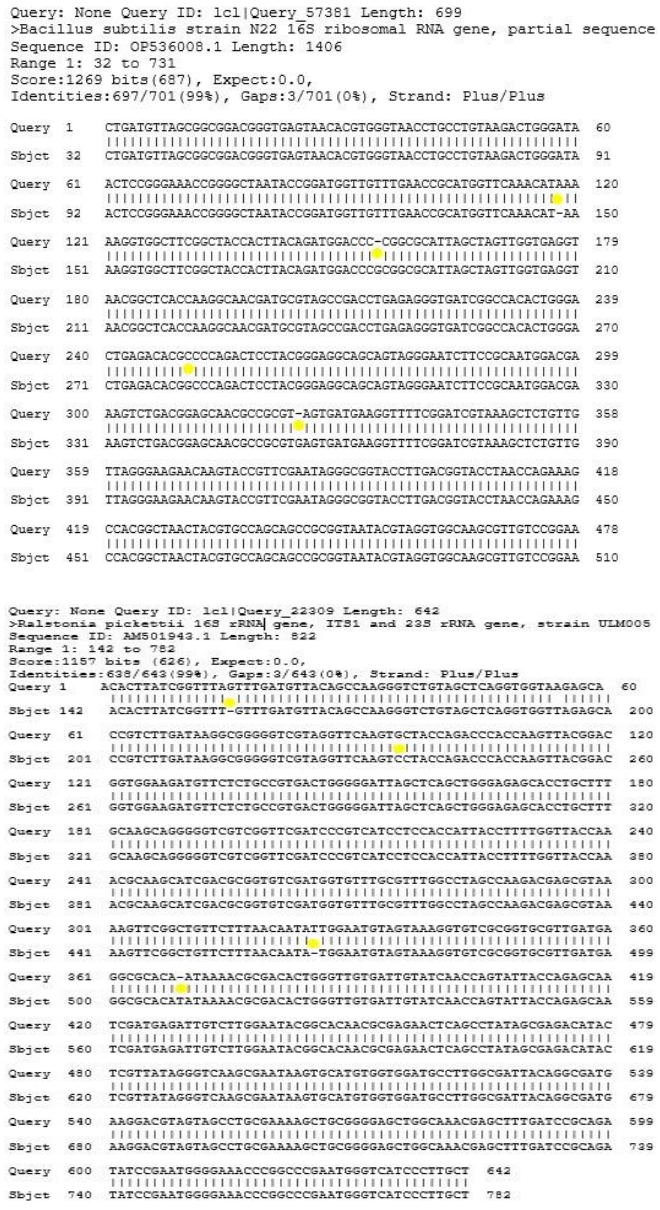

Figure 6. Nucleotide sequence alignment of the *16S rRNA* gene of the Z7 bacteria with the closest match to the *Bacillus subtilis* N22 registered in GenBank.

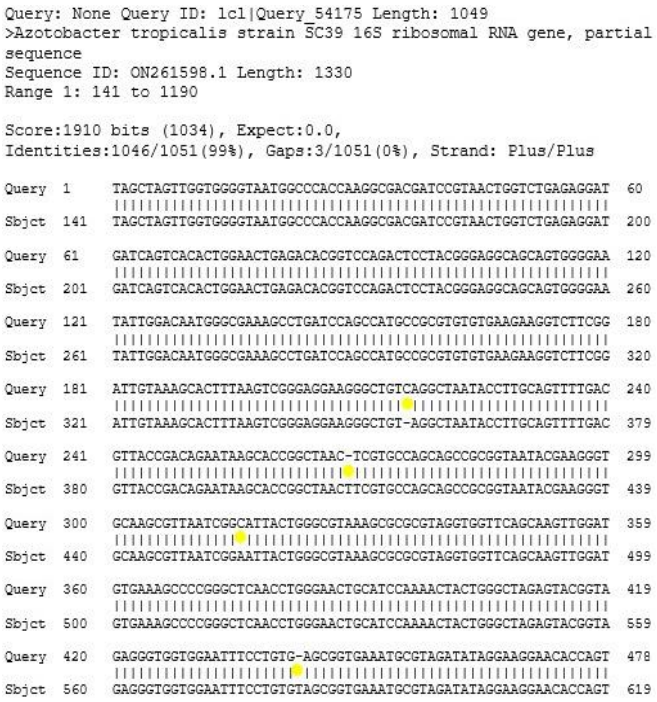

Figure 7. Nucleotide sequence alignment of the *16S rRNA* gene of the Z11 bacteria with the closest match to the *Azotobacter tropicalis* SC39 registered in GenBank.

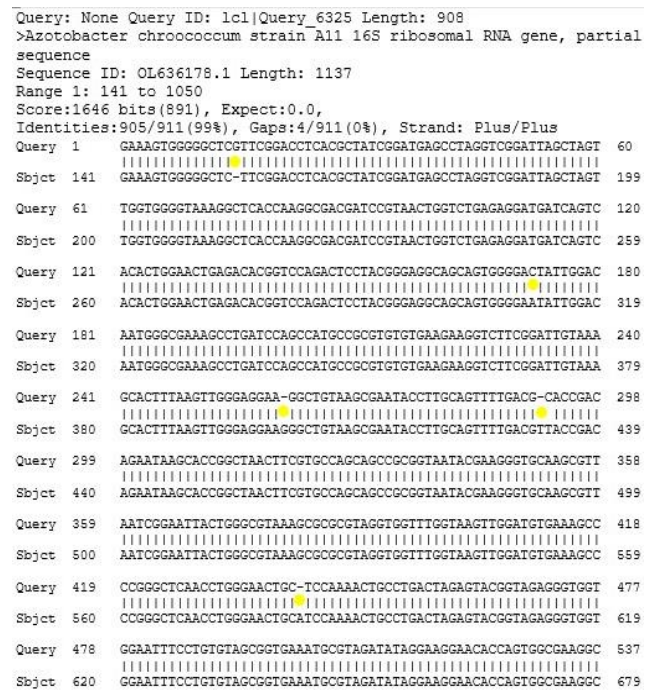

Figure 8. Nucleotide sequence alignment of the *16S rRNA* gene of the Z16 bacteria with the closest match to the *Azotobacter chroococcum* A11 registered in GenBank.

The slight difference in the nucleotide sequence in terms of deletion, addition and substitution did not affect the diagnosis of the four types of bacteria individually and indicates the occurrence of a genetic mutation that occurred within the requirements of evolutionary adaptation, and this is consistent with what was indicated by Horton and Taylor (2023).

***Conclusion*:** The results of this study will increase our knowledge of the types of rhizobacteria found in Iraqi soils that are most active in producing the plant growth hormone IAA, so that the results of this study can be used to enrich future scientific experiments related to the use of new bacteria, represented by rhizobacteria, to produce environmentally sustainable agricultural crops.

***Acknowledgment*:** The authors are very grateful to the College of Agriculture and Forestry, University of Mosul for providing all the assistance and help which helped to finalize this research with a good quality of work.

***Author's contributions Statement*:** Conceptualization and Methodology, M.A.H. and N.I.K.A.; Investigation, M.A.H. and F.A.A.; Data Curation, M.A.A. and N.I.K.A.; Writing – Original Draft reparation, M.A.H. and F.A.A.; Writing – Review & Editing, M.A.A. and N.I.K.

***Conflict of interest*:** The authors declare no conflict of interest.

***Funding*:** No.

***Availability of data and material*:** Data will be made available on request.

***Informed consent*:** N/A

***Consent for publication*:** All authors submitted consent to publish this research article in JGIAS.

***SDG's Addressed*:** Zero hunger, Responsible consumption and production.

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